NEURAL ACTIVITY IN AMYGDALA SUBDIVISIONS EVOKED BY PERIODIC AND/OR SINGLE ENRICHING EXPERIENCES IN ADOLESCENT RATS

by

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Abstract

Adolescence is often a stressful period of time marked with low cognitive control, high emotionality, and increased risk taking behaviors that expose adolescents to a variety of novel and emotional experiences. Environmental enrichment (EE) provides a way to manipulate sensory, motor, and social experiences through interaction with same sex conspecifics in a setting often containing a variety of objects, ramps, and platforms. Enrichment in adolescence influences brain development including the amygdala and related behavior in emotionally arousing situations. In the amygdala, the lateral amygdala (LA) is the primary input zone that sends processed stimulus- derived information to the anterior basolateral amygdala (aBLA) and posterior basolateral amygdala (pBLA), which provide context to that information. Processed information is then sent to the central nucleus of the amygdala (CeA) for output to target brain areas. The purpose of this study was to examine how enriching experiences during adolescent development might differentially affect neuron density and neural activation in LA, aBLA, pBLA and CeA. There were four experimental groups of Long Evans adolescent rats with a history of enrichment or not between postnatal day (PND) 25 and 48, and with a final enrichment exposure or not prior to sacrifice on PND 49. After sacrifice, brain tissue was processed to visualize the neural activity marker c-FOS, and alternate Nissl sections were stained with thionin to discern total neuron densities within subdivisions of the amygdala. In the LA, there was a 24% greater density of neurons with a history of enrichment compared to non enriched rats ($p< 0.001$) suggesting periodic enrichment may spare neurons in LA. Rats with a history of EE displayed decreases in c-FOS+ neurons in LA -52%, pBLA -33%, and CeA -32% regions (all $p< 0.05$), with no significant difference in aBLA. The data suggests that rats may become accustomed to

periodic EE exposure, which can explain less neural activation in amygdala of rats with an enrichment history. Experiments designed to examine how a history of EE affects performance in other behavioral tasks might help explain the difference in LA neuron density as well as differential neural activation in amygdala subdivisions discovered in this study.

Keywords: lateral amygdala, anterior basolateral amygdala, posterior basolateral amygdala, central nucleus of the amygdala, environmental enrichment.

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Neural Activity in Amygdala Subdivisions Evoked by Periodic and/or Single Enriching Experiences in Adolescent Rats

Adolescence is a period of increased exposure to emotional experiences that may impact brain development and responses to emotional stimuli. Novel experiences in adolescence have a demonstrated influence on risk taking behavior, problem solving, learning and memory (Steinberg, 2007; Forgays & Forgays, 1951; Mora, Segovia & del Arco, 2007; Simpson & Kelly, 2007; van Praag, Kempermann & Gage, 2000). Studying the effect of emotional experiences on adolescents has been the focus of past research (Sturman & Moghaddam, 2011, Mora et al., 2007) especially utilizing the environmental enrichment (EE) paradigm (Simpson et al., 2007; Koe, Ashokan & Mitra, 2016; Fernandez, Escorihuela & Castellano, 1997). A rodent model of enrichment includes social interaction with novel same-sex conspecifics, and sensorimotor stimulation including multiple ramps, objects, and platforms which generates behavioral responses to emotionally arousing stimuli (Okuda, Tatsumi, Makinodan, Yamauchi, Kishimoto & Wanaka, 2009; Simpson et al., 2011; van Praag et al., 2000). The effects of EE have been found to induce changes in the biochemistry as well as the morphology of the brain by strengthening synapses, increasing dendritic branching, and increasing overall brain weight and size (Mcdonald,1984; Mohammed et al., 2002). Ultimately, EE can produce a lasting effect on learning and memory, problem solving, risk taking, exploratory behavior, and reduced fear by means of environmental novelty (Ali, Wilson & Murphy, 2009; Forgays et al., 1951, Cain, Green & Bardo, 2006; Matsuda, Ehara, Nakadate, Yoshimoto & Ueda, 2018; Simpson et al., 2011; Ashokan, Hege & Mitra, 2016). Enrichment paradigms can be used to study how novel, emotional experiences in adolescence can impact the amygdala and influence subsequent behavior following enrichment.

The major divisions of the amygdala are the lateral amygdala (LA), basolateral amygdala (BLA), and Central nucleus of the amygdala (CeA), which process incoming stimuli and integrate information with previously stored knowledge (Kim, Pignatelli, Xu, Itohara & Tonegawa, 2016; Yang & Wang, 2017; Janak & Tye, 2015; Mcdonald, 1984; Miller & Urcelay, 2007; Peggs & Gaillard, 2018; Sah, Faber, Lopez & Power, 2003; Ali et al., 2009). The BLA is often further broken down into anterior basolateral amygdala (aBLA) and posterior basolateral amygdala (pBLA) (Kim et al., 2016; Matsuda et al., 2018; Mcdonald, 1984; Miller et al., 2007; Yang et al., 2017). The LA is the primary input zone of auditory, somatosensory, and visual stimuli, which works to collect information from multiple brain regions outside of the amygdala (Hariri, 2005; McDonald, 1984; Pitkänen, Savander & LeDoux, 1997). Once the LA has filtered incoming stimuli, it provides input to aBLA and pBLA, which integrates and relays information to the central nucleus of the amygdala CeA. This information received by CeA, the output structure, is sent to other areas of the brain to produce behavioral responses (Pitkänen et al., 1997; Hariri, 2005; Mcdonald, 1982). The purpose of this investigation was to observe how a history of enrichment may differentially impact neural density and activation in each subdivision of the amygdala, as well as how a final exposure to enrichment may affect neural activation in the amygdala.

Adolescence

Adolescence is marked by novel and emotional experiences which may occur suddenly or expectedly, and can have a significant impact on brain development and behavior (Blakemore & Choudhury, 2006; Hammerslag & Gulley, 2016; Koe et al., 2016). Such experiences strongly influence an organism's development of emotional responses and risk-taking behaviors as well as problem-solving abilities and learning. Especially in cases of

adversity, the impact of various experiences on emotion and behavior can be long-lasting (Koe et al., 2016). The period of adolescence can be researched in humans and can be modelled across many species, with a rat model being particularly efficacious (Spear, 2000). The period of adolescence in rats translates from weaning around postnatal day (PND) 21 to, at latest, early adulthood at PND 60 (Lynn & Brown, 2009; Tirelli, Laviola, & Adriani, 2003). Studying adolescent behavior with rat as a model is favorable due to the ability to control the nature of their interactions and environment, as well as track development.

Based on epidemiological data, human adolescence when compared to other ages accounts for a period of increased reckless behavior, sensation seeking, and risk taking involving hazardous driving, substance misuse, and unprotected sex (Spear, 2000; Macrì, Adriani, Chiarotti, & Laviola, 2002; Ernst & Hardin, 2005; Sturman et al., 2011). Alongside increased risk-taking oftentimes comes the emergence of psychological disorders like substance misuse, anxiety and depression (Hammerslag et al., 2016). With less mature cognitive control systems, and an interest to engage in risky behaviors, adolescence is a period of increased vulnerability for humans as well as other species (Steinberg, 2007; Stansfield & Kirstein, 2006). In both rats and humans, adolescents exhibit considerably higher impulsivity and a marked desire for novel sensations and experiences, which decreases with development (Sturman et al., 2011; Hammerslag et al., 2016). Adolescent rats are able to habituate more efficiently to novel stimuli than adults and, in turn, to novel situations (Stansfield et al., 2006). Macrì et al. (2006) showed that adolescence is a period where motivation to explore novel environments surpasses any potential risk-taking consequences.

During adolescence, changes in the brain allow development of decision-making habits and cognitive processing through development of neural circuitry. Adolescent behavior is laced with impulsivity and extensive, risky exploratory behavior in novel situations. This suggests that decisions are based more on emotion or sensation than reason (Stansfield et al., 2006; Steinberg, 2007) and also suggests involvement of neural circuits important for the control of emotion. Two distinct neural networks that develop rapidly during adolescence are the: socioemotional network, responsible for risk taking, and the cognitive control network, which is responsible for executive functioning and decision making (Steinberg, 2007). Trying to discern the timeline for development of these regions is important to understand attraction to novelty and risk taking behaviors that seem to preside for the bulk of adolescence (Steinberg, 2007). Development of the cognitive control system in adolescence is shaped by increased executive functioning in the prefrontal cortex which boosts the ability to control emotional reactivity and impacts risk taking behavior (Sturman et al., 2011).

As adolescents try new things, explore, and take risks, there are a lot of opportunities for informal learning and formation of memories. In addition to novelty seeking, adolescence is also a period of biological, social, and psychological changes. During this period, the brain undergoes vast changes contributing to behaviors, emotions, and individual motivation (Blakemore et al., 2006; Spear, 2000; Lynn et al., 2009; Ernst et al., 2005). Specific changes to mammalian brains during adolescence include the formation of new synaptic connections, which produces a greater density of connections, followed by synaptic pruning, where remaining connections are strengthened with use and in turn become more efficient (Blakemore et al., 2006; Mora et al., 2007; van Praag et al., 2000). A multitude of brain

regions are affected by development throughout adolescence shown by global increases in white matter continuing into adulthood, but dramatic decreases in grey matter throughout adolescence (Blakemore et al., 2006). Synaptic pruning and increased myelination that occurs in adolescence results in faster reaction times and more efficient transmission of neural impulses. This type of evidence of neural plasticity was first pointed out by Cajal (c. 1894) who proposed one's environment has an impact on brain development and function (Mora et al., 2007; DeFelipe, 2006). He noted changes in neural connections and that these changes are more robust in the young and diminish with age (Mora et al., 2007; DeFelipe, 2006; Sampedro-Piquero, Begega, Zancada-Menendez, Cuesta & Arias, 2013). Since then, exposure to novel environments have been demonstrated to produce particularly profound effects in adolescent compared to adult brains and behavior (Sampedro- Piquero et al., 2013; Stansfield et al., 2006; Steinberg, 2007). Thus, adolescence serves as a particularly salient period of time for the study of neural development and changes in brain as well as behavior.

Environmental Enrichment

Experimental EE paradigms provide scientists with an opportunity to measure the effects of interactions and experiences in unique environments on adolescent development. In the 1940s, controlled EE studies began with research performed by Donald O. Hebb who found that animals reared in enriched environments had superior problem solving abilities than those reared in standard housing due to the opportunity for perceptual learning (Forgays et al., 1951). An enriched setting includes opportunities for sensory and motor stimulation alongside social interaction with novel and familiar same-sex conspecifics (Mora et al., 2007; Okuda et al., 2009; Simpson et al., 2011; van Praag et al., 2000). This type of environment is far more stimulating than a standard environment with the ability to generate behavioral

responses to emotionally arousing stimuli (Mora et al., 2007; Okuda et al., 2009; Simpson et al., 2011; van Praag et al., 2000). For example, EE provides the opportunity to for an animal to interact and explore in novel environments utilizing multiple ramps, toys, and platforms on set schedules (Okuda et al., 2009; Simpson et al., 2011; van Praag et al., 2000). For adolescent animals, typical enrichment schedules employ continuous or periodic enrichment over a four to eight-week period and EE is typically introduced immediately following weaning (Simpson et al., 2011). Although a single exposure to enrichment can produce potent effects, periodic enrichment is important for producing lasting effects (Simpson et al., 2011; van Praag et al., 2000; Mora et al., 2007). Because adolescence is filled with multiple experiences that will continue to shape decisions and behavior long term, measuring the modifications to brain structure and function as a result of EE can be used to model development.

EE can have a robust impact on exploratory behavior, problem solving, memory, learning, and novelty seeking in adolescent rats (Forgays et al., 1951, Mora et al., 2007; Simpson et al., 2011; van Praag et al., 2000). The primary reason for these effects is due to the unique opportunity that EE provides for informal learning. Exposure to EE affects sensory, emotional, and cognitive systems as well as inducing modifications in cell morphology and synaptic plasticity of rodent brains (Ali et al., 2009; Mohammed et al., 2002; Mora et al., 2007; Simpson et al., 2011; van Praag et al., 2000). Enrichment promotes neural plasticity leading to the creation of stronger more efficient connections within and between brain structures allowing for increased neural activity and an enhanced capacity for learning. Housing animals in enriched environments, or allowing for periodic EE experiences can enhance the potential for memory formation and learning by affecting activity in

hippocampal formation circuits in both adolescent and adult rats (Pavelka, 2018; van Praag et al., 2000, Simpson et al., 2011). The effects of EE have also been studied in the human hippocampus where exposure to exercise increased neurogenesis and angiogenesis (Clemenson, Deng & Gage, 2015). While the effects of enrichment on learning and memory are well-researched, the effect of enrichment on amygdala circuitry is less researched and is the primary focus of the current study.

Amygdala

The amygdala is often described as a small almond shaped structure located deep within the temporal lobe, and it is important for learning and assessing emotional significance of events in our lives (Phelps, 2004; Sah et al., 2003). Structures within the amygdaloid complex and circuitry are responsible for processing emotion-evoking input and producing emotion-based behaviors such as fear, pain, hope, and desire. Though the human experience of emotion may be different, the structure and function of the amygdala is highly conserved across species which allows for more comparative neurology than for some brain structures (Janak et al., 2015; Phelps, 2004). Being able to appreciate research on amygdala circuitry in other species allows us to study function and make predictions about behaviors.

The main sub regions of the amygdala, as usually described for rats, include the LA, BLA, and CeA, which are associated with processing fear and anxiety among other emotions (Kim et al., 2016; Yang et al., 2017; Janak et al., 2015; Mcdonald, 1984; Miller et al., 2007; Peggs et al., 2018; Sah et al., 2003; Ali et al., 2009; see Figure 2). Amygdaloid circuitry is considered reciprocal in that information is not sent in a unidirectional fashion, rather it is a highly complex web of interconnectedness (Pitkänen et al., 1997; Sah et al., 2003, see also Figure 3). Hariri (2005) explains that neural signals derived from stimuli either bypass

sensory cortices and travel straight from the thalamus through amygdaloid circuitry quickly with low resolution following a “low road” (p. 21) which lacks a level of detail, or slowly with higher resolution following a “high road” (p. 21) path with input of well-processed information from sensory cortices. When input is processed at a higher resolution, it travels through the thalamus and sensory cortices collecting detail and this subsequently leads to much slower processing with slower behavioral response. However, for example, if confronted with potentially dangerous stimuli, input may bypass the sensory cortices and travel through thalamus to the amygdala much quicker with lower resolution which produces faster behavioral responses.

The LA is the first structure in the amygdala circuit, and it receives most of the sensory afferent input from auditory, visual, gustatory, somatosensory, thalamic nuclei, hippocampal formation and prefrontal cortex then sends information accordingly to other BLA nuclei in the amygdala (Yang et al., 2017; Janak et al., 2015; Hariri, 2005; Sah et al., 2003). The LA plays a pivotal role in receiving and integrating stimuli before it sends information to other nuclei. The LA is also considered an essential component of the amygdala underlying fear conditioning memory (Sah et al., 2003; Yang et al., 2017; Ashokan et al., 2016).

The BLA complex has been found to play an important role in forms of emotional memory (Sah et al., 2003) and anxiety (Yang et al., 2017; Ashokan et al., 2016) making it a major integration zone for anxiety evoking stimuli. The BLA can be considered the relay station in the amygdala complex that gathers and integrates information from other areas of the brain responsible for reward, memory. It then sends the integrated negative or positive valenced information to output areas like the CeA. Due to differences in cell type, large size,

and shape of the BLA, it is commonly divided into anterior and posterior sections (Kim et al., 2016; Matsuda, et al., 2018; Mcdonald, 1984; Miller et al., 2007; Yang et al., 2017) or medial and basal sections (Janak et al., 2015; Sah et al., 2003). For the purposes of the current study, the BLA is divided into anterior BLA (aBLA) and posterior BLA (pBLA). Both aBLa and pBLA project directly to the hippocampal CA1 region to establish monosynaptic connections (Yang et al., 2017; Huff, Emmons, Narayanan & LaLumiere, 2016) with the pBLA projection being denser than the aBLA projection (Yang et al., 2017). Greater activation in aBLA to CA1 hippocampal circuitry has been shown to correlate with increased anxiety and social deficits while the pBLA to CA1 connections are more related to increases in emotion modulated spatial memory (Yang et al., 2017). In an experiment that utilized negative and positive valenced stimuli, Kim et al. (2016) found more activation in aBLA when negative stimuli such as foot shock and aversive olfactory stimuli were presented which elicited avoidance. The pBLA was more activated by positive stimuli such as water, sugar, and other desired stimuli/ reward which elicited positive behavioral responses (Kim et al., 2016). This research suggests that there are distinct populations of neurons in the BLA that may become active selectively to positive or negative valence stimuli. Along the same lines, research in fear conditioning, where anterior and posterior sections of the BLA were inactivated, showed that aBLA plays a bigger role in fear conditioning than pBLA (Goosens, 2001) suggesting pBLA may play a bigger role in more positive valence circuitry. Golgi stain studies have revealed that the organization of neurons in the pBLA is precise and highly ordered when compared to aBLA and other amygdala circuitry which may indicate that it processes information differently (McDonald, 1984).

The central nucleus of the amygdala (CeA) receives input from both the LA and the BLA (Pitkänen et al., 1997; Hariri, 2005; Mcdonald, 1982). What is processed in the different input zones is sent to the CeA where information converges and efferent projections are sent to other areas in the brain (Pitkänen et al., 1997; Sah et al., 2003; Mcdonald, 1982). Lesion research has also shown that CeA plays a role in the output of processed fear conditioning (Goosens et al., 2001). The CeA is considered the main output region of the amygdala and has extensive projections to the brainstem fear effector areas which evoke visceral adaptive responses as well as the hypothalamus for autonomic and endocrine responses (Yang et al., 2017; Ernst et al., 2005; Mcdonald, 1982; Hariri, 2005). In an urgent situation, the CeA can send output information very quickly to other regions of the brain for prompt behavioral responses (Pitkänen et al., 1997)

Enrichment and the Amygdala

The amygdala is responsible for the processing emotions, anxiety, and as such, plays a role in risk-taking behaviors. During adolescence, the propensity for risk-taking and novelty seeking, regardless of the potential for harm, is influenced by a weak harm- avoidant system (amygdala), a strong reward system (nucleus accumbens), and a slower developing control center (prefrontal cortex) (Ernst et al., 2005). Furthermore, the amygdala is crucial in tasks requiring attention, the development of memories, and regulating how incoming stimuli can be perceived through an emotional lens (Phelps, 1997; Pitkänen et al., 1997). Previous literature suggests that repeated exposure to EE for even just a short period of time can decrease stress and attenuate anxiety experiences that originate in the amygdala (Ali et al., 2009, Ashokan et al., 2016). Enriched animals exhibit decreased stress responses (Mora et al., 2007), and are less emotionally reactive in novel situations (Simpson et al., 2011) due to

changes in the amygdala. Enriched housing often decreases anxiety and fear, promoting more efficient exploration and increased habituation to novel environments (Simpson et al., 2011, Sturman et al., 2011; Wellman et al., 2017).

Over time, exposure to EE, especially during adolescence, can equip developing animals with neuroprotective circuitry in the amygdala that decreases the likelihood of exhibiting anxiety behaviors in social settings and novel environments. Furthermore, rats repeatedly exposed to enrichment also displayed lower levels of neural activity at a 24 h post-experiment sacrifice than controls in a multitude of brain regions, namely, the amygdala (Mohammed et al., 2002, Simpson et al., 2011). This suggests that enriched rodents were more capable of processing and adapting to a novel and perhaps stressful experiences as compared to controls. In a study conducted by Mohammed et al. (2002) manipulation of the environment can result in morphological changes in both the neurons and synaptic connections between neurons of the nuclei within the amygdala of rats. Observed changes due to enrichment include increased dendritic branching and cell proliferation (Mcdonald, 1982; Mcdonald, 1984). These changes equip the brain for more complex development of behavior through emotion-based decision making within the amygdala (Mohammed et al., 2002). A study done by Ashokan et al. (2016) showed a decrease in the number of primary and secondary dendritic spines in rats exposed to a chronic immobilization stress condition that were housed in EE settings, versus no decrease in dendritic spines for rats reared without EE exposed to the chronic immobilization stress condition. Through repeated exposure to EE, the amygdala's extrinsic and intrinsic neural connections would likely become more efficient while creating more purposeful dendritic branching through developmental mechanisms of synaptic pruning (Peggs et al., 2018).

When considering the role of emotion-based decision making and the benefits of enrichment in adolescence, repeated exposure to EE could decrease a fear response through mechanisms of neural plasticity in young rats. Analysis of fear conditioning in rats has suggested that long-term synaptic plasticity of inputs to the amygdala underlies the acquisition and perhaps storage of the fear memory (Sah et al., 2003). There are multiple physiological similarities between animal and human fear, and fear conditioning is seen as relevant to the genesis of anxiety disorders in humans which provides additional support for the study of the roots of fear and emotion (Sah et al., 2003).

Current Study

Understanding amygdala circuitry and how it can be shaped by experiences is important, especially when considering the range of emotionally charged experiences that occur during adolescent development. Enrichment has the potential to have potent short term and long term effects on the amygdala that can be measured. The purpose of the current study was to investigate how activation of amygdala circuitry may differ in adolescent rats when they experience routine, periodic EE and/or a single exposure to EE. There were four groups in the study. The group of rats with a history of EE and a single last exposure to EE is denoted by (EEEE), while the group with a history of EE and no last exposure to EE is denoted by (EENO). The rats with just a single last exposure to EE is denoted by (NOEE), and control rats with no exposure to EE are denoted as (NONO) (see Figure 1).

Brain development during adolescence is highly impacted by context and environmental factors. One way to track neural development in animals is to discern where there are increases or decreases in neural densities in particular brain regions. Utilizing Nissl staining methods enables a comparison across structures and experimental conditions to

identify changes in the number of total neurons. This is of particular interest when tracking the effects of a history of enrichment through adolescent development. It was hypothesized that total neuron density within the amygdala subdivisions (La, aBLA, pBLA, CeA) would differ in animals with a history of enrichment (EEEE & EENO groups) versus those with no history of enrichment (NOEE & NONO groups) (Hypothesis 1). A history of enrichment is important for increasing neural efficiency and thus a history of EE could change the efficiency of circuits and lead to the paring down of neural connections.

EE may impact expression of the immediate early gene, *c-fos*, which is rapidly and transiently expressed after synaptic stimulation (Sagar, Sharp & Curran, 1988). The *c-fos* gene encodes for c-FOS protein which serves as a general marker for tracking differences in activation following EE experiences. The c-FOS protein is induced in neurons after extracellular stimulation by trophic substances and neurotransmitters and particularly by Ca^{2+} influx into the cell through NMDA receptor channels or voltage gated calcium channels following depolarization in the cell (Chaudhuri, 1997). Quantifying the activation of c-FOS protein is an efficient method to view changes in neural activation with periodic exposure to EE as well as after a single exposure to EE. In the current study, c-FOS immunoreactivity indicates neural activity within the amygdala if increased c-FOS protein expression is observed, or an increase in quantity of c-FOS positive neurons.

Based on findings from relevant research (e.g., Ali et al., 2009, Peggs et al., 2018; Mohammed et al., 2002), it was hypothesized that a history of EE (EEEE group) would attenuate the proportion of activated c-FOS+ neurons in subdivisions of the amygdala (La, aBLA, pBLA, CeA) when compared to just a single last exposure to EE (NOEE group) (Hypothesis 2). Less neural activation was expected in the history of EE condition due to

lower reactivity and increased ability to deal with stress when exposed to enrichment overtime (Mora et al., 2007; Ali et al., 2009), and higher neural activation was predicted for the NOEE group with a single exposure to EE that increases activation in brain regions including the amygdala (Ali et al., 2009; Peggs et al., 2018). Taking into account the expected attenuating effect of EE on the amygdala, it was also hypothesized that the proportion of c-FOS+ neurons in rats with a history of EE (EEEE group) versus non enriched controls (NONO) would be similar (Hypothesis 3).

The amygdala as a whole plays an important role in fear memory and fear learning (Wellman et al., 2017). Based on previous literature (Ali et al., 2009, Peggs et al., 2018) it was hypothesized that a single novel exposure to EE (NOEE group) would evoke higher levels of neural activation in the LA input zone of the amygdala when individually compared to aBLA and pBLA (Hypothesis 4). The integrative nature of the BLA plays a key role in mediating fear and associated emotions (Ashokan et al., 2016; Ali et al., 2009, Wellman et al., 2017), and thus the effects of a single EE exposure may not be as potent in aBLA and pBLA as compared to the highly targeted input zone, LA.

Materials and Methods

Experimental Design and Archival Brain Tissue

Archival brain tissue collected from 12 male and 12 female Long-Evans hooded rats that were bred and cared for in the Arts and Sciences Animal Facility at Appalachian State University provided the samples used for quantification of Nissl staining and c-FOS immunoreactivity. Care of the rats and all experimental procedures were approved by the Institutional Animal Care and Use Committee at Appalachian State University (Protocol #15-02, M. C. Zrull, PI, see Appendix). While living, rats were randomly assigned to conditions

following a 2 X 2 experimental design (Periodic Enrichment X Single Exposure Enrichment). The four experimental conditions included one group that received periodic enrichment and a last exposure to enrichment (EEEE), and a second group that received periodic enrichment and no last exposure to enrichment (EENO). The third and fourth groups were housed in standard conditions receiving no periodic enrichment but were picked up and put down on the days corresponding to periodic enrichment for EEEE and EENO groups to control for handling effects. While the third group experienced a single exposure to enrichment prior to sacrifice (NOEE), the fourth group received no enrichment experience at all and served as the baseline control for the study (NONO). Periodic enrichment took place between postnatal day (PND) 25 and PND 48 for a total of 18 sessions. The single exposure to EE occurred on PND 49 just prior to sacrifice. The purpose of the single exposure to enrichment was to evoke measurable activity in brain structures just prior to sacrifice.

Enrichment took place in a 45.7 X 48.3 X 78.7 cm (w X d X h) wooden frame enclosure with a 1/2-in. hardware cloth sides and top that was closed by a wood framed, hardware cloth latched door. The floor of the cage was covered with aspen bedding. A wide array of assorted color, shape, size, and textured objects were placed within the enrichment cage on platforms and the the cage floor. Other objects hung from the mesh ceiling and ramps inside the cage. The platforms were located at 14.0, 24.8, 43.2, and 61.0 cm above the floor of the cage and were made accessible to animals via ramps. In each session that rats experienced, objects were swapped out and placement of objects was changed with a total of four different stimulating novel environments (see Figure 4). In the enrichment cages, the rats were able to interact with familiar and novel same sex conspecifics with whom they were not regularly housed. Each EE session took place for a duration of 1.5 h.

Histology

The archival tissue utilized in this study was harvested after rats were placed in quiet, dark conditions for 90min following a single EE exposure (EEEE and NOEE conditions) or being taken from the home cage (EENO and NONO conditions) and after a lethal injection of sodium pentobarbital (100 mg/kg b.w., ip). Upon the absence of corneal and tail reflex, each subject was then intracardially perfused using phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 10 mM phosphate buffer (PB). Following the perfusion, brains were collected and fixed in a 10% sucrose, 4% paraformaldehyde solution at 4 °C for a week and then stored in a PB and 0.05% sodium azide solution until the tissue was processed. The brain tissue was cut into 50 μm sagittal sections using a Vibratome Series 1000 Sectioning System and processed using floating section immunohistochemistry (IHC) to stain for the c-FOS protein as a neural marker of activation.

On the first day of IHC, floating sections were rinsed in PBS (2 x 5 min) and subsequently incubated in 15% goat serum with 0.25% Triton-X in PBS for 1 h. The sections were then placed in anti-c-FOS made in rabbit (Cell Signaling Technology 2250S) for about 40 hours. On the second day of IHC, floating sections were rinsed in PBS (6 x 10 min) and incubated for 1 h in biotinylated goat anti-rabbit secondary antibody (Vector Labs) and then rinsed again in PBS (3 x 10 min). Sections were floated in a peroxidase-labeled avidin-biotin complex for 1 h (Vector Labs) and rinsed again in PBS (2 x 10 min). Sections were exposed to the VIP enzyme substrate (Vector Labs) for 2 min and then placed into chilled PBS until the sections were mounted onto gel-coated slides. After drying, the sections were dehydrated in graded ethanols, cleared with toluene, and cover- slipped with Permount (Fisher).

Alternate sections were processed to stain all cell bodies with thionin in order to observe neural densities and cytoarchitecture in conjunction with c-FOS stained sections.

Microscopy and Data Analysis

With respect to the c-FOS protein, neuronal activity was defined by stained neuron nuclei that were quantified using a systematic counting method to compare levels of activation between enriched and unenriched rats with or without a history of periodic enrichment. Sections were first viewed using a Nikon Eclipse microscope and Plan 4 objective to localize regions of the amygdala and then with a Plan 10 objective to capture 1024 x 768 pixel image with a 1.3 megapixel PixeLink digital camera and photo capture software. The structures within the amygdala (i.e., LA, aBLA, pBLA and CeA), were distinguished with the help of thionin stained alternate sections and two atlases of the rat brain (Paxinos & Watson, 1998; Pellegrino, Pellegrino, & Cushman, 1979). Adobe Photoshop CS4 was used to overlay gridlines on to each 1024 x 786- pixel images of a particular structure. Based on cytology, regional shape, and structure size, a specified number of 200 x 200 μm boxes were randomly selected (2 for LA, 4 for pBLA, 3 for aBLA, 3 for CeA), and the activated neurons within the selected boxes were counted. Neural nuclei were considered activated based upon darkness of staining. Generally, neurons that stain darker have higher levels of activation, as indicated by greater concentration of c-FOS, than those that are lighter (Sagar et al.,1988; Chaudhuri, 1997). Only the darkest neural nuclei in comparison to other nuclei within sampling frames were included in the counts and represented in the final dataset. Cells in each brain section across all structures in the study were counted by two experimenters and were checked for inter-rater reliability. For the 756 c-FOS counting frames, the inter-rater reliability was r=0.88. The counts were averaged

together for analysis. Although each experimental condition originally consisted of six brains in each group for a total of 24 brains, the total number of brains included in each group can be found in Table 1, which details the number of cases available per group as well as mean c-FOS activation. Data from certain cases were excluded in experimental analysis due to errors in perfusion, tissue damage in processing, or staining anomalies.

In addition to being used in defining regions of the amygdala and orienting the experimenter to where structures of interest were located, neurons in the amygdala of alternate thionin stained sections were counted. These Nissl sections were quantified by counting neurons within amygdala subdivisions of sections neighboring the c-FOS sections. Neurons were identified by parameters including the presence of transparent cell body, a distinguishable membrane, the presence of a nucleus, and larger size than opaque small glial cells and other objects detailed in Figure 5 (García-Cabezas, John, Barbas & Zikopoulos, 2016). A variety of small and large neurons were identified and counted in the same fashion as the c-FOS stained neural nuclei were counted in order to compare the total activated to the total neuron densities within each amygdala region of interest. Cells in each brain section across all structures in the study were counted by two experimenters and were checked for inter-rater reliability. For the 756 Nissl counting frames, the inter-rater reliability was r=0.93. The counts were averaged together for analysis.

Results

Nissl Neuron Density with a History of Enrichment

Due to the nature of environmental enrichment to promote neural sparing or neural pruning, it was predicted that there would be a difference in total neuron density between animals with or without a history of enrichment (Hypothesis 1). The differences in neuron

density with respect to Nissl staining between rats with a history of enrichment (EEEE and EENO groups) and those without (NOEE and NONO groups) across amygdala subdivisions were compared using a 1-way ANOVA. The results show that neuron density in the LA input region differed significantly between the history of EE and no history of EE conditions: $F(1, 16) = 6.83$, $p = .019$, $\eta^2 = 0.299$, in that there was a 24% greater mean density of neurons in the LA of animals with a history of EE ($M = 103.3$, $SD = 20.4$) as compared to in animals without EE ($M = 82.7$, $SD = 9.8$). This difference between groups is also notably marked by a large effect size. In contrast, there were no significant differences found in density of neurons between the history of enrichment group ($M = 64.2$, $SD = 5.8$) and no history of enrichment groups ($M = 66.0$, $SD = 6.6$) for aBLA, $F(1, 20) = 0.46$, $p = .504$, $\eta^2 = 0.0226$. For pBLA, there was no difference between history of EE ($M = 75.0$, $SD = 7.0$) and no history of EE ($M = 72.0$, $SD = 5.0$), $F(1, 20) = 1.32$, $p = .264$, $\eta^2 = 0.0620$. Similarly, there was no difference with a history of enrichment ($M = 136.1$, $SD = 11.6$) and no history of enrichment ($M = 127.0$, $SD = 18.6$) for CeA, $F(1, 18) = 1.60$, $p = .222$, $\eta^2 = 0.0817$ (see also Figure 6). Thus, Hypothesis 1, which predicted that neuron density in rats with a history of enrichment versus no history would be different, was supported for the LA input zone but not supported for other subdivisions of the amygdala (aBLA, pBLA, and CeA).

Enrichment Effects on Proportion of c-FOS+ Neurons

For tests of the remaining hypotheses, a proportion of c-FOS+ neurons was computed by dividing mean c-FOS neuron count by the total neurons stained with thionin in each amygdala subdivision (see Figure 7). The proportion of neural activation observed in the amygdala with a history of enrichment and final enriching experience (EEEE) was expected to be lower than the proportion of activation observed in the amygdala with just a single

exposure to enrichment (NOEE) due to the attenuating effects of EE on the amygdala (Hypothesis 2). This hypothesis was tested using a 2-factor ANOVA to partition variance, which showed what variability was due to the effects of independent variables (EE history, last EE) and what was due to error. Subsequently, t-tests were run to compare proportion of c-FOS+ neurons in amygdala subdivisions (LA, aBLA, pBLA, and CeA) across the groups of interest. Type I error for purposes of determining outcome of the hypothesis tests was set at .05 for each *a priori* test. The results suggest a smaller proportion of c-FOS+ neurons in the EEEE group as compared to the NOEE group for LA, pBLA, and CeA: 52% decrease in LA, 32% decrease in pBLA, and 33% decrease in CeA. The largest difference in activation between the EEEE group (M= 0.10, SD= 0.03) and NOEE group (M= 0.17, SD= 0.04) was observed in LA, t (14) =-3.75, p= .001, d= 1.98. The second largest difference in activation with a history and last experience of enrichment (M= 0.13, SD= 0.04) versus a single last exposure to enrichment (M= 0.18, SD= 0.03) was in pBLA, t (18) =-2.14, p=.023, d= 1.40. A smaller difference in activation between EEEE (M= 0.10, SD= 0.04) and NOEE (M= 0.14, SD= 0.03) groups were observed in the CeA, t (16) =-1.77, p=.048, d= 1.13. However, there was no difference in proportion of c-FOS positive neurons in the aBLA of EEEE (M= 0.22, SD= 0.03) and NOEE (M= 0.21, SD= 0.05) groups, t (18) =0.49, p=.316, d= 0.20 (see also Table 1 and Figure 7). Therefore, Hypothesis 2 was supported mostly in that there was a significantly smaller proportion of c-FOS activated neurons in all amygdala subdivisions except for aBLA.

It was predicted that neural activation in the EEEE group and the NONO group would be similar due to the attenuating effects of a history of enrichment (Hypothesis 3). This hypothesis was tested by using a 2-factor ANOVA to partition variance, which showed what

variability was due to the effects of independent variables (EE history, last EE) and what was due to error. Paired samples t-tests were conducted to compare data across amygdala subdivisions (LA, aBLA, pBLA, and CeA) taking into account the two groups of interest. Type I error for purposes of determining outcome of the hypothesis tests was at .05 for each *a priori* test. The results show that there was no significant difference between the proportion of c-FOS positive neurons in EEEE (M= 0.10, SD= 0.01) and NONO (M= 0.09, SD= 0.01) groups with respect to: LA, t (14) =0.67, p=.257, d= 0.447. There was also no difference in neural activation in pBLA between EEEE (M= 0.13, SD= 0.04) and NONO (M= 0.12, SD= 0.05) groups, t (18) =0.70, p=.247, d= 0.221. Furthermore, in the CeA subdivision, the EEEE (M= 0.10, SD= 0.04) animals did not differ from NONO (M= 0.08, SD= 0.03) animals either, t (16) =1.10, p=.143, d= 0.567 (see also Table 1 and Figure 6). However, there was a statistically significant difference between c-FOS activation between EEEE (M= 0.22, SD= 0.05) and NONO (M= 0.16, SD= 0.06) in aBLA, t (18) =1.92, p=.035, d= 1.086 (see also Table 1 and Figure 6). The hypothesis is partially supported in that a history of EE seems to attenuate neural activation in all subdivisions of the amygdala except for aBLA.

Single Exposure to Enrichment Effects in LA and BLA

A single exposure to enrichment is novel experience, thus it may evoke a higher proportion of neural activation in different amygdala subdivisions due to incoming stimuli first converging on the LA and then diverging to the aBLA and pBLA. It was predicted that higher levels of neural activation would be observed in the LA when individually compared to aBLA and pBLA with respect to the NOEE group (Hypothesis 4). This hypothesis was tested by using independent sample t-tests to compare the proportion of c-FOS positive neurons in LA and aBLA as well as in LA and pBLA within the NOEE group. Type I error

for purposes of determining outcome of the *a priori* hypothesis tests was set at .05 for each test. The results showed that this hypothesis was not supported, and the activation was actually greater in aBLA, t (8) =-1.17, p=.137, *d*= 0.883 than in the LA input zone. Activation was also greater in pBLA, t (8) =-0.47, p=.324, *d*= 0.283, as compared to LA. The hypothesis was not supported in that there was less neural activation in the LA input zone than both the aBLA and pBLA areas of integration. The group means for the NOEE group can be found in Table 1 and Figure 7.

Discussion

Adolescence is a period of time where there is increased risk-taking (Spear, 2000; Macrì et al., 2002; Ernst et al., 2005; Sturman et al., 2011), exposure to new novel experiences and environments (Blakemore et al., 2006; Hammerslag et al., 2016; Koe et al., 2016), as well as decreased cognitive control in decision making (Steinberg et al., 2007). Due to lasting negative effects of adversity experienced early in adolescence on a behavioral and emotional level (Koe et al., 2016), it is important to understand ways to mitigate the impact and better equip those affected by these common emotionally salient experiences. The current study examined how neuron density and neural activation in subdivisions of the amygdala may be shaped and changed by exposure to a history of EE, or exposure to EE prior to sacrifice.

In Hypothesis 1, a difference in neuron density between animals with a history of enrichment and those without was predicted due to background research that suggests a history of enrichment can alter connections in the amygdala (Mora et al., 2007) as well as promote cell proliferation and repress cell death in the amygdala (Okuda et al., 2009). There was no difference found between rats with and without a history of enrichment in the aBLA,

pBLA, and CeA. However, there was a 24% difference in neuron density counted in the LA in rats with a history of EE.

Through normal adolescent development, there is typically a loss of gray matter especially in the temporal lobe during late adolescence (Sturman et al., 2011), where the amygdala is located. Subsequently, there is usually an increase in white matter which suggests more efficient connections between neurons with axonal sprouting and increased myelination of those pertinent connections (Sturman et al., 2011). With these typical developmental changes in mind, the difference in neuron density observed in the LA input region of the amygdala with a history of EE could suggest neural sparing where these neurons would usually be pruned in development. EE has been found in previous research to promote neural protection via cell survival, synaptogenesis, and increased neurogenesis (Mora et al., 2007). The increased neural density in the LA region with a history of EE suggests that each subdivision of the amygdala (LA, aBLA, pBLA, CeA) are differentially affected by EE. One possible reason for increased neural density in enriched animals only in the LA could be due to its importance as an input zone for all incoming stimuli. Over time, the exposure to EE periodically could have equipped the rats with superior initial processing of the incoming stimuli in LA. With more continuous exposure to EE and increased activation of LA neurons, the subdivision might have to become more efficient, thus sparing neurons to receive and process the high volume of incoming information to then send to other areas in the amygdala.

Previous literature on how a history of EE impacts functional activation in brain regions have be explored through the rapid and transient expression of the immediate early gene *c-fos* by growth factors and neurotransmitters (Sagar et al., 2009). In the current study, a

proportion of c-FOS expression was computed by dividing the mean average c-FOS+ neurons by the neuron density from average Nissl counts for each amygdala subdivision. In Hypothesis 2, it was proposed that a history of EE would attenuate the proportion of activated c-FOS+ neurons in subdivisions of the amygdala as compared to just a single last exposure to EE. The hypothesis was supported in that the percentage of c-FOS+ neurons decreased in LA -52%, pBLA -32%, and CeA -33%. This aligns with previous research which shows lower levels of c-FOS neural activation in animals with a history of EE that were exposed to an open-field task compared to those without a history of EE (Nikolaev, Kaczmarek, Zhu, Winblad, & Mohammed, 2002). Decreased locomotor activity and freezing behavior as well as increased adaptation/habituation are behavioral changes that correlate with changes in amygdala activation. These changes suggest decreased arousal, emotionality, and anxiety (Mohammed et al., 2002; Sztainberg et al., 2010; Matsuda et al., 2018). Furthermore, enriched rats take less time than non enriched rats to recover from stressful experiences (Mohammed et al., 2002).

The data in the current study suggests that aBLA is differentially affected by EE compared to other amygdala subdivisions. The aBLA was the only region that did not significantly differ in neural activation between the EEEE group and NOEE group. Due to increased c-FOS production being linked to fear conditioning (Janak et al., 2015), it makes sense that activation in aBLA may by greater given that it is primarily responsible for processing anxiety and social interactions, while pBLA relates more to emotional modulated spatial memory (Yang et al., 2017; Goosens, 2001). Results from the current study suggest that in aBLA, with a history of EE or not, a last exposure to enrichment just prior to sacrifice was potent enough to evoke neural reactions.

In Hypothesis 3, it was predicted that the proportion of c-FOS+ neurons in rats with a history of EE versus non enriched controls would be similar. This prediction was made due to previous research that showed attenuating effects of EE on the amygdala and subsequent behavioral responses (Mcdonald, 1982; Mcdonald, 1984; Mohammed et al., 2002; Ashokan et al, 2016). For example, Ashokan and colleagues (2016) showed that only two weeks of exposure to EE mitigated the propensity to develop increased anxiety following chronic immobilization stress tasks paired with less dendritic branching of neurons in areas of the basolateral amygdala. Most all of the subdivisions in the amygdala (LA, pBLA, and CeA) showed that there was no significant difference between control rats and those in the history of EE group. These results suggest that a history of EE was able to mitigate the effects of the last exposure to EE which is done to evoke activity prior to sacrifice. The aBLA showed a significant increase in neural activation with a history and last exposure to EE when compared to non enriched controls. This means that EE history was not as effective in mitigating expression of c-FOS protein located in neurons of the aBLA just prior to sacrifice. As stated previously, the aBLA has specific functions that differ from pBLA and other subdivisions of the amygdala and thus could be impacted differentially by EE.

Due to the nature of c-FOS protein being rapidly and transiently expressed (Sagar et al., 1988), and the LA receiving all of the incoming stimuli input, Hypothesis 4 predicted that a single novel exposure to EE would evoke higher levels of neural activation in LA as compared to aBLA and pBLA integration zones. The data showed that c-FOS neural activation was actually greater in aBLA and pBLA than LA with respect to the NOEE group, though the difference was not statistically significant, and effect sizes were small. Since increased c-FOS expression in the BLA are associated with anxiety related behavior

(Matsuda et al., 2018), the results from this current study show again that a history of enrichment is important in mitigating the effects on the expression of c-FOS that the last exposure to EE prior to sacrifice evokes.

The different type of information and ways in which LA, aBLA and pBLA process stimuli could also play a role in the slight variation of neural activation with a single enriching experience. While the LA processes a substantial amount of incoming stimuli, the aBLA and pBLA receive and integrate this stimulus for context (Pitkänen et al., 1997). The The BLA as a whole has also been found to be crucial for mediating neurotransmitter release while influencing memory for inhibitory avoidance learning, which is stored and sent to other modalities by the CeA (Nikolaev et al., 2002). Previous research has shown a single short episode of EE was able to reduce anxiety-like behavior exhibited by in maternally separated rats (Koe et al., 2016). Neurally, this exposure to separation without EE led to degeneration of dendrites and increased spine density in the BLA long into adulthood (Koe et al., 2016). Similar results were found in a stress inducing paradigm where higher levels of brain-derived neurotrophic factor (BDNF), which promote cell survival and functions associated with synaptic plasticity, increased dendritic branching, and spine density in the BLA was observed in the no history of enrichment and exposure to an immobilization stress task (Ashokan et al., 2016). This research is suggesting that the protective effects of a history of EE can expand across other contexts. When considering differences in evoked activity in LA, aBLA, and pBLA in Hypothesis 4, the single last exposure to EE in rats without a prior history of EE in the current study seems to be evoking higher levels of stress and anxiety denoted especially by the slight increased neural activation in the aBLA and pBLA subdivisions.

Limitations and Future Directions

The current study was conducted with a relatively small group of animals, and in order to get a clearer more balanced representation of what is happening in each subdivision, groups of about eight animals would be beneficial. Furthermore, without comparing EE effects to an impoverished group of animals in isolation with no social interaction or any exposure to contact, conclusions cannot be made based on what would happen if there is no social interaction or any stimulation. Extending the study into other contexts and settings may also be insightful. In order to understand the long term effects of EE after being periodically exposed to enrichment, it would be interesting to extend the study to look at how enriched rats versus non enriched rats react in different tasks designed to evoke some level of fear/anxiety such as an elevated plus maze (Ali et al., 2009), or an open field test (Nikolaev et al., 2002) to see if enriched rats are more equipped to deal with potential fear-evoking environments.

Because it is difficult to determine exactly what kind of cells are activated when the c-FOS staining method is used, counterstaining techniques such as staining for glutamate or GABA as well as testing for cortisol levels and/or BDNF levels in each group would provide more definitive results about what is going on in amygdala subdivisions in response to EE. Further, because it is hard to tell what is happening day- to- day during development and testing of neural tissue occurs just after sacrifice in studies like this thesis research, neural changes are only able to be interpreted as immediate after effects rather than at various points in time. Extending the study to younger rats to see how EE may affect early neural development, and also extending this research beyond adolescence could provide insight on how enrichment effects in adolescence may affect juvenile animals and carry through to

adulthood. Further research is needed to fully understand the exact mechanisms of how EE affects different subdivisions in the amygdala and the roles of each subdivision.

Summary and Conclusions

A history of EE impacts neural activation and neuron density in the amygdala subdivisions differentially. When analyzing c-FOS neural activation, it is important to take into account total neurons in each subdivision to compute an accurate proportion of c-FOS+ neurons per subdivision within each experimental group. Within each subdivision of the amygdala, periodic exposure to EE was beneficial in reducing proportion of activation and thus equipping the brain with more efficient connections. Some form of enrichment can be utilized in every day life encouraging novel experiences and increased social interaction as it has been shown beneficial for development. A single exposure to EE evokes higher levels of neural activation and based on previous literature, this translates to increased anxiety and stress responses (Koe et al., 2016; Ashokan et al., 2016; Yang et al., 2017). The effect of the last experience of EE on activation is mitigated with a history of enrichment which seems to provide neural protective effects and attenuation of anxiety. Understanding the intricacies of anatomical structure and function in the amygdala and its connections to other regions of the brain is of great importance in considering implications for effectively integrating emotional information in decision making (Sturman et al., 2011), as well as understanding a wide range of emotional diseases and emotion-associated memory impairment, including anxiety, depression and Alzheimer's disease (Sah et al., 2003; Yang et al., 2017).

References

Ali, A. E. A., Wilson, Y. M., & Murphy, M. (2009). A single exposure to an enriched environment stimulates the activation of discrete neuronal populations in the brain of the fos-tau-lacZ mouse. *Neurobiology of Learning and Memory*, *92*(3), 381–390. https://doi.org/10.1016/j.nlm.2009.05.004

Ashokan, A., Hegde, A., & Mitra, R. (2016). Short-term environmental enrichment is sufficient to counter stress-induced anxiety and associated structural and molecular plasticity in basolateral amygdala. *Psychoneuroendocrinology*, *69*, 189–196. https://doi.org/10.1016/j.psyneuen.2016.04.009

Blakemore, S.-J., & Choudhury, S. (2006). Development of the adolescent brain: implications for executive function and social cognition. *Journal of Child Psychology and Psychiatry*, *47*(3–4), 296–312. https://doi.org/10.1111/j.1469-7610.2006.01611.x

Cain, M. E., Green, T. A., & Bardo, M. T. (2006). Environmental enrichment decreases responding for visual novelty. *Behavioural Processes*, *73*(3), 360–366. https://doi.org/10.1016/j.beproc.2006.08.007

Chaudhuri, A. (1997). Neural activity mapping with inducible transcription factors. Neuro Report, 8(13): iii-vii.

Clemenson, G. D., Deng, W., & Gage, F. H. (2015). Environmental enrichment and neurogenesis: from mice to humans. *Current Opinion in Behavioral Sciences*, *4*, 56 62. https://doi.org/10.1016/j.cobeha.2015.02.005

DeFelipe, J. (2006). Brain plasticity and mental processes: Cajal again. Nat. Rev. Neurosci. 7, 811–817.

Ernst, M., Pine, D. S., & Hardin, M. (2005). Triadic model of the neurobiology of motivated

behavior in adolescence. *Psychological Medicine*, *36*(03), 299. https://doi.org/10.1017/S0033291705005891

Fernandez-Teruel, A., Escorihuela, R. M., Castellano, B., Gonzalez, B., & Tobena, A. (1997). Neonatal Handling and Environmental Enrichment Effects on Emotionality, Novelty/Reward Seeking, and Age-Related Cognitive and Hippocampal Impairments: Focus on the Roman Rat Lines. *Behavior Genetics, 27*(6), 513-526.

Forgays, D. G., & Forgays, J. W. (1951). The nature of the effect of free-environmental experience in the rat. Journal of Comparative Physiological Psychology, 45(4), 322-8.

García-Cabezas, M. Á., John, Y. J., Barbas, H., & Zikopoulos, B. (2016). Distinction of Neurons, Glia and Endothelial Cells in the Cerebral Cortex: An Algorithm Based on Cytological Features. *Frontiers in Neuroanatomy*, https://doi.org/10.3389/fnana.2016.00107

Goosens, K. A. (2001). Contextual and Auditory Fear Conditioning Are Mediated by the Lateral, Basal, and Central Amygdaloid Nuclei in Rats. *Learning & Memory*, *8*(3), 148–155. https://doi.org/10.1101/lm.37601

Hammerslag, L. R., & Gulley, J. M. (2016). Sex differences in behavior and neural development and their role in adolescent vulnerability to substance use. *Behavioural Brain Research*, *298*, 15–26. https://doi.org/10.1016/j.bbr.2015.04.008

Hariri, A. R. (2015). Looking inside the disordered brain: An introduction to the functional neuroanatomy of psychopathology. Sunderland, MA, U.S.A: Sinauer Associates.

Huff, M. L., Emmons, E. B., Narayanan, N. S., & LaLumiere, R. T. (2016). Basolateral amygdala projections to ventral hippocampus modulate the consolidation of footshock, but not contextual, learning in rats. *Learning & Memory*, *23*(2), 51–60.

https://doi.org/10.1101/lm.039909.115

Janak, P. H., & Tye, K. M. (2015). From circuits to behaviour in the amygdala. *Nature*, *517*(7534), 284–292. https://doi.org/10.1038/nature14188

Kim, J., Pignatelli, M., Xu, S., Itohara, S., & Tonegawa, S. (2016). Antagonistic negative and positive neurons of the basolateral amygdala. *Nature Neuroscience*, *19*(12), 1636–1646. https://doi.org/10.1038/nn.4414

Koe, A. S., Ashokan, A., & Mitra, R. (2016). Short environmental enrichment in adulthood reverses anxiety and basolateral amygdala hypertrophy induced by maternal separation. *Translational Psychiatry*, *6*(2), e729–e729. https://doi.org/10.1038/tp.2015.217

Lynn, D. A., & Brown, G. R. (2009). The ontogeny of exploratory behavior in male and female adolescent rats (Rattus norvegicus). *Developmental Psychobiology*, *51*(6), 513–520.https://doi.org/10.1002/dev.20386

Macrì, S., Adriani, W., Chiarotti, F., & Laviola, G. (2002). Risk taking during exploration of a plus-maze is greater in adolescent than in juvenile or adult mice. *Animal Behaviour*, *64*(4), 541–546. https://doi.org/10.1006/anbe.2002.4004

Matsuda, W., Ehara, A., Nakadate, K., Yoshimoto, K., & Ueda, S. (2018). Effects of environmental enrichment on the activity of the amygdala in microcephalic rats exposed to a novel open field: *Fos* activation in microcephalic rats. *Congenital Anomalies*, *58*(1), 16–23. https://doi.org/10.1111/cga.12228

McDonald, A. J. (1982). Cytoarchitecture of the central amygdaloid nucleus of the rat. *The Journal of Comparative Neurology, 208*(4), 401-418.

McDonald, A. J. (1984). Neuronal organization of the lateral and basolateral amygdaloid nuclei in the rat. *The Journal of Comparative Neurology*, *222*(4), 589–606. https://doi.org/10.1002/cne.902220410

Miller, S. S., & Urcelay, G. P. (2007). The Central Amygdala Joins the Lateral Amygdala in the Fear Memory Party. *Journal of Neuroscience*, *27*(9), 2151–2152. https://doi.org/10.1523/JNEUROSCI.5460-06.2007

Mohammed, A. H., Zhu, S. W., Darmopil, S., Hjerling-Leffler, J., Ernfors, P., Winblad, B., Bogdanovic, N. (2002). Environmental enrichment and the brain. In *Progress in Brain Research* (Vol. 138, pp. 109–133). Elsevier. https://doi.org/10.1016/S0079-6123(02)38074-9.

Mora, F., Segovia, G., & del Arco, A. (2007). Aging, plasticity and environmental enrichment: Structural changes and neurotransmitter dynamics in several areas of the brain. *Brain Research Reviews*, *55*(1), 78–88. https://doi.org/10.1016/j.brainresrev.2007.03.011

Nikolaev, E., Kaczmarek, L., Zhu, S. W., Winblad, B., & Mohammed, A. H. (2002). Environmental manipulation differentially alters c-Fos expression in amygdaloid nuclei following aversive conditioning. *Brain Research*, *957*(1), 91–98. https://doi.org/10.1016/S0006-8993(02)03606-5

Okuda, H., Tatsumi, K., Makinodan, M., Yamauchi, T., Kishimoto, T., & Wanaka, A. (2009). Environmental enrichment stimulates progenitor cell proliferation in the amygdala. *Journal of Neuroscience Research*, *87*(16), 3546–3553. https://doi.org/10.1002/jnr.22160

Peggs, C. A., & Gaillard, C. E. (2018). A Novel Experience of Environmental Enrichment

Affects the Basolateral and Lateral Amygdala Differently in Adolescent Rats, 8.

Phelps, E. A., & Anderson, A. K. (1997). Emotional memory: What does the amygdala do? Current Biology, 7(5). doi:10.1016/s0960-9822(06)00146-1

Phelps, E. A. (2004). Human emotion and memory: interactions of the amygdala and hippocampal complex. *Current Opinion in Neurobiology*, *14*(2), 198–202. https://doi.org/10.1016/j.conb.2004.03.015

Pitkänen, A. (1997). Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. *Trends in Neurosciences*, *20*(11), 517–523. https://doi.org/10.1016/S0166-2236(97)01125-9

Sagar, S. M., Sharp, F. R., & Curran, T. (1988). Expression of c-fos Protein in Brain: Metabolic Mapping at the Cellular Level. *Science, New Series*, *240*(4857), 1328–1331.

Sah, P., Faber, E. S. L., Lopez De Armentia, M., & Power, J. (2003). The Amygdaloid Complex: Anatomy and Physiology. *Physiological Reviews*, *83*(3), 803–834. https://doi.org/10.1152/physrev.00002.2003

Sampedro-Piquero, P., Begega, A., Zancada-Menendez, C., Cuesta, M., & Arias, J. L. (2013). Age-dependent effects of environmental enrichment on brain networks and spatial memory in Wistar rats. *Neuroscience*, *248*, 43–53. https://doi.org/10.1016/j.neuroscience.2013.06.003

Simpson, J., & Kelly, J. P. (2011). The impact of environmental enrichment in laboratory rats-Behavioural and neurochemical aspects. *Behavioural Brain Research*, *222*(1), 246–264. https://doi.org/10.1016/j.bbr.2011.04.002

Spear, L. P. (2000). The adolescent brain and age-related behavioral manifestations.

Neuroscience & Biobehavioral Reviews, *24*(4), 417–463. https://doi.org/10.1016/S0149-7634(00)00014-2

Stansfield, K. H., & Kirstein, C. L. (2006). Effects of novelty on behavior in the adolescent and adult rat. *Developmental Psychobiology*, *48*(1), 10–15. https://doi.org/10.1002/dev.20127

Steinberg, L. (2007). Risk Taking in Adolescence: New Perspectives From Brain and Behavioral Science. *Current Directions in Psychological Science*, *16*(2), 55–59. https://doi.org/10.1111/j.1467-8721.2007.00475.x

Sturman, D. A., & Moghaddam, B. (2011). The neurobiology of adolescence: Changes in brain architecture, functional dynamics, and behavioral tendencies. *Neuroscience & Biobehavioral Reviews*, *35*(8), 1704–1712. https://doi.org/10.1016/j.neubiorev.2011.04.003

Sztainberg, Y., Kuperman, Y., Tsoory, M., Lebow, M., & Chen, A. (2010). The anxiolytic effect of environmental enrichment is mediated via amygdalar CRF receptor type 1. *Molecular Psychiatry*, *15*(9), 905–917. https://doi.org/10.1038/mp.2009.151

Tirelli, E., Laviola, G., & Adriani, W. (2003). Ontogenesis of behavioral sensitization and conditioned place preference induced by psychostimulants in laboratory rodents. *Neuroscience & Biobehavioral Reviews, 27*(1-2), 163-178. https://doi.org/10.1016/S0149-7634(03)00018-6

van Praag, H., Kempermann, G., & Gage, F. H. (2000). Neural consequences of environmental enrichment. Nature Reviews: Neuroscience, 1, 191-198.

Wellman, L. L., Fitzpatrick, M. E., Hallum, O. Y., Sutton, A. M., Williams, B. L., & Sanford, L.D. (2017). The basolateral amygdala can mediate the effects of fear

memory on sleep independently of fear behavior and the peripheral stress response. *Neurobiology of Learning and Memory*, *137*, 27–35. https://doi.org/10.1016/j.nlm.2016.11.004

Yang, Y., & Wang, J.-Z. (2017). From Structure to Behavior in Basolateral Amygdala-Hippocampus Circuits. *Frontiers in Neural Circuits*, *11*. https://doi.org/10.3389/fncir.2017.00086

Table 1. *Mean c-FOS+ Neural Activation in Amygdala Subdivisions of Interest for Each Experimental Group*

	EE-EE		EE-NO		NO-EE		NO-NO	
	n	*M(SD)*	*n*	*M(SD)*	*n*	*M(SD)*	*n*	*M(SD)*
LA	6	9.0 (2.3)	4	8.5 (3.0)	4	14.7 (3.1)	4	6.9 (2.0)
aBLA	6	13.8 (3.5)	5	8.5 (4.6)	6	13.9 (2.7)	5	10.2 (4.2)
pBLA	6	9.4 (1.8)	5	6.4 (2.8)	6	13.4 (1.8)	5	8.4 (3.8)
CeA	5	13.9 (4.7)	4	8.1 (1.2)	6	17.2 (3.3)	5	10.5 (3.6)

		Last Exposure	
		EE	NO
History	EE	EEEE	EENO
	NO	NOEE	NONO

Figure 1. Experimental design 2X2 within subjects factorial design. Groups in the study with a history of EE (EEEE and EENO) and/or a last exposure to EE prior to sacrifice (NOEE and EEEE). The (NONO) group serves as a baseline control for the study.

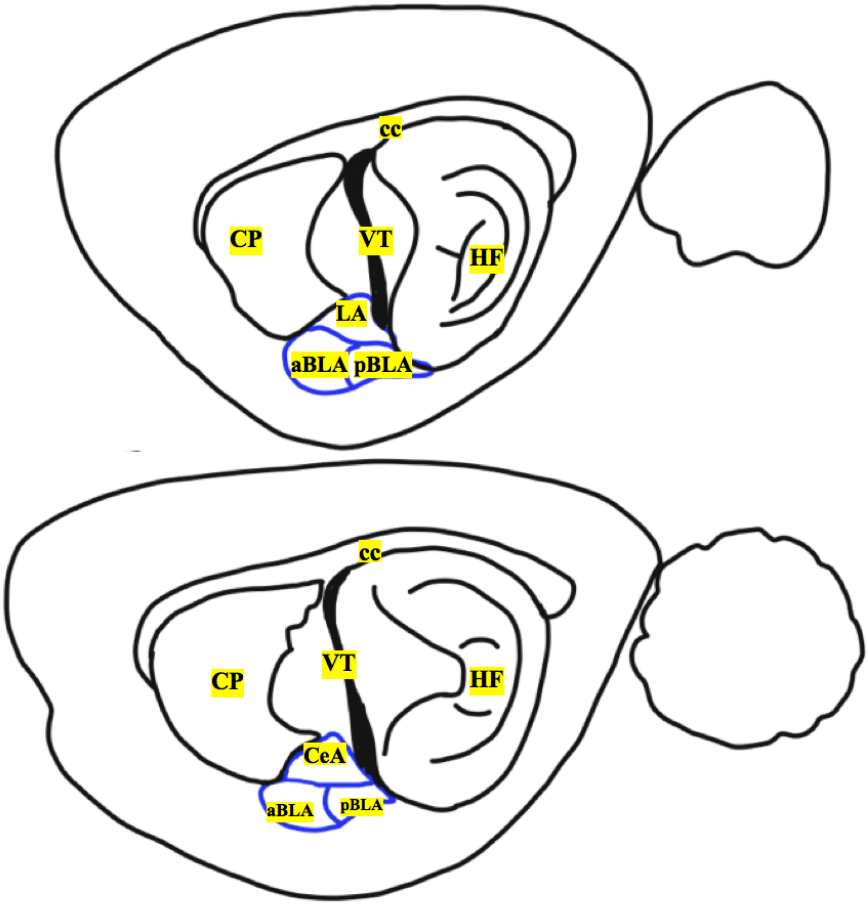

Figure 2. Areas of the brain (LA, aBLA, pBLA & CeA) counted for c-FOS+ neurons as well as thionin stained total neurons in each subdivision. Drawings were adapted from Paxinos & Watson (4th ed.), 1998, Academic Press. *Abbreviations:* anterior basolateral amygdala (aBLA), corpus callosum (cc), central nucleus of the amygdala (CeA), caudate putamen (CP), hippocampal formation (HF), lateral amygdala (LA), posterior basolateral amygdala (pBLA), ventricle (VT).

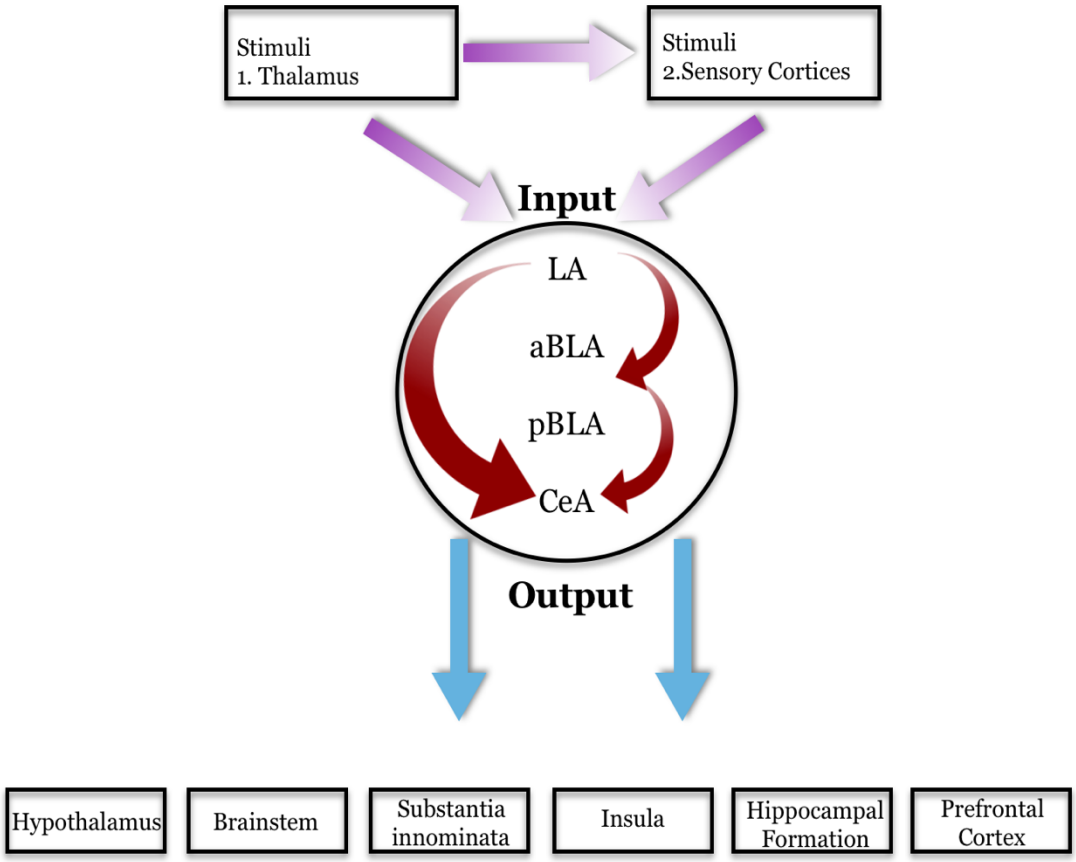

Figure 3. Circuit diagram, adapted from Hariri (2015), detailing incoming stimuli that can either travel from the thalamus then sensory cortices, or directly from the thalamus. The LA receives the input and sends information to the aBLA and pBLA which integrate the information. The LA can also send information directly to CeA bypassing the aBLA and pBLA completely. The CeA receives information that it processes for output to target areas including the hypothalamus, brainstem, substantia innominata, insula, hippocampal formation, and prefrontal cortex. Output to these areas produce physiological and/ or behavioral responses. *Abbreviations:* anterior basolateral amygdala (aBLA), central nucleus of the amygdala (CeA), lateral amygdala (LA), posterior basolateral amygdala (pBLA).

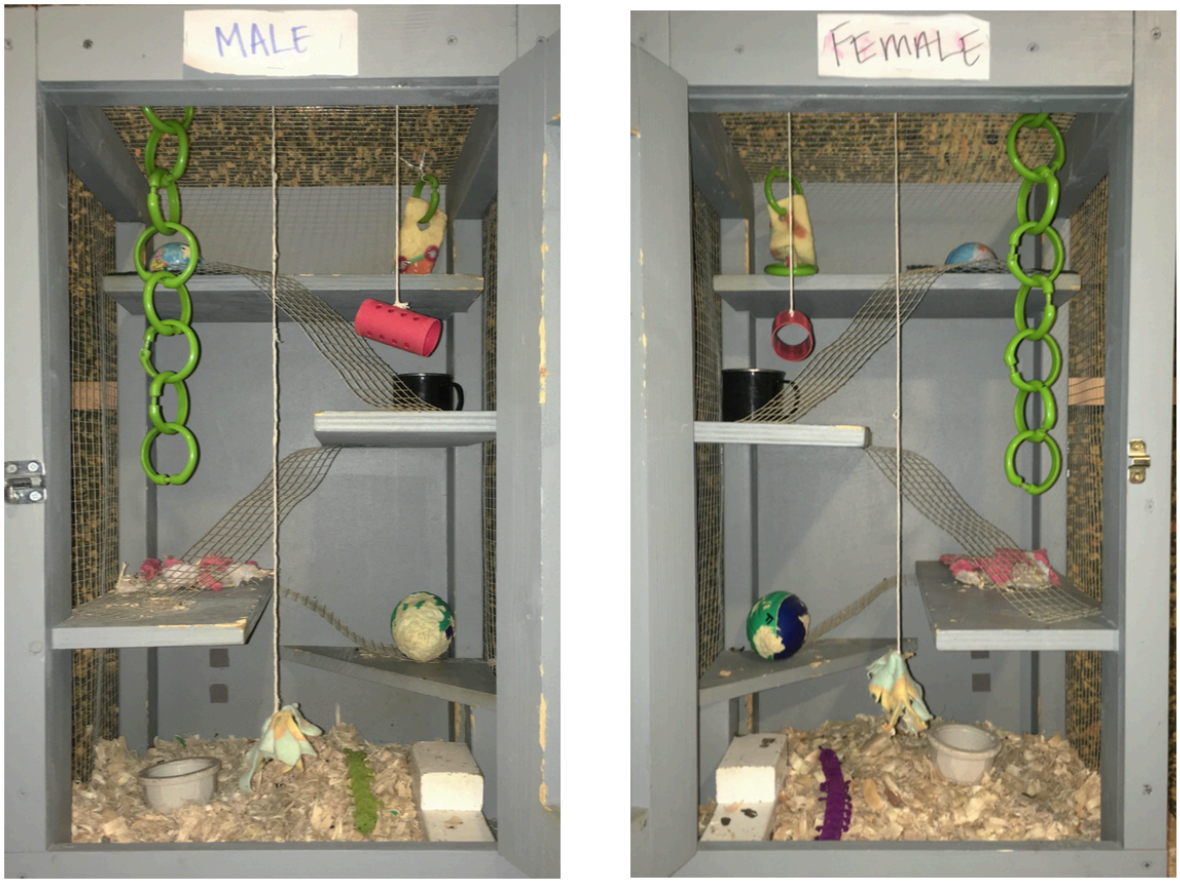

Figure 4. One of the enrichment cage set-ups with various objects, ramps, and platforms. Objects vary in size, texture, shape, and color. The male enrichment cage and female enrichment cages always mirrored each other in object set-up. Enrichment included same sex conspecifics that were both familiar and novel. The set-ups changed from session to session to maintain a degree of novelty in the environment.

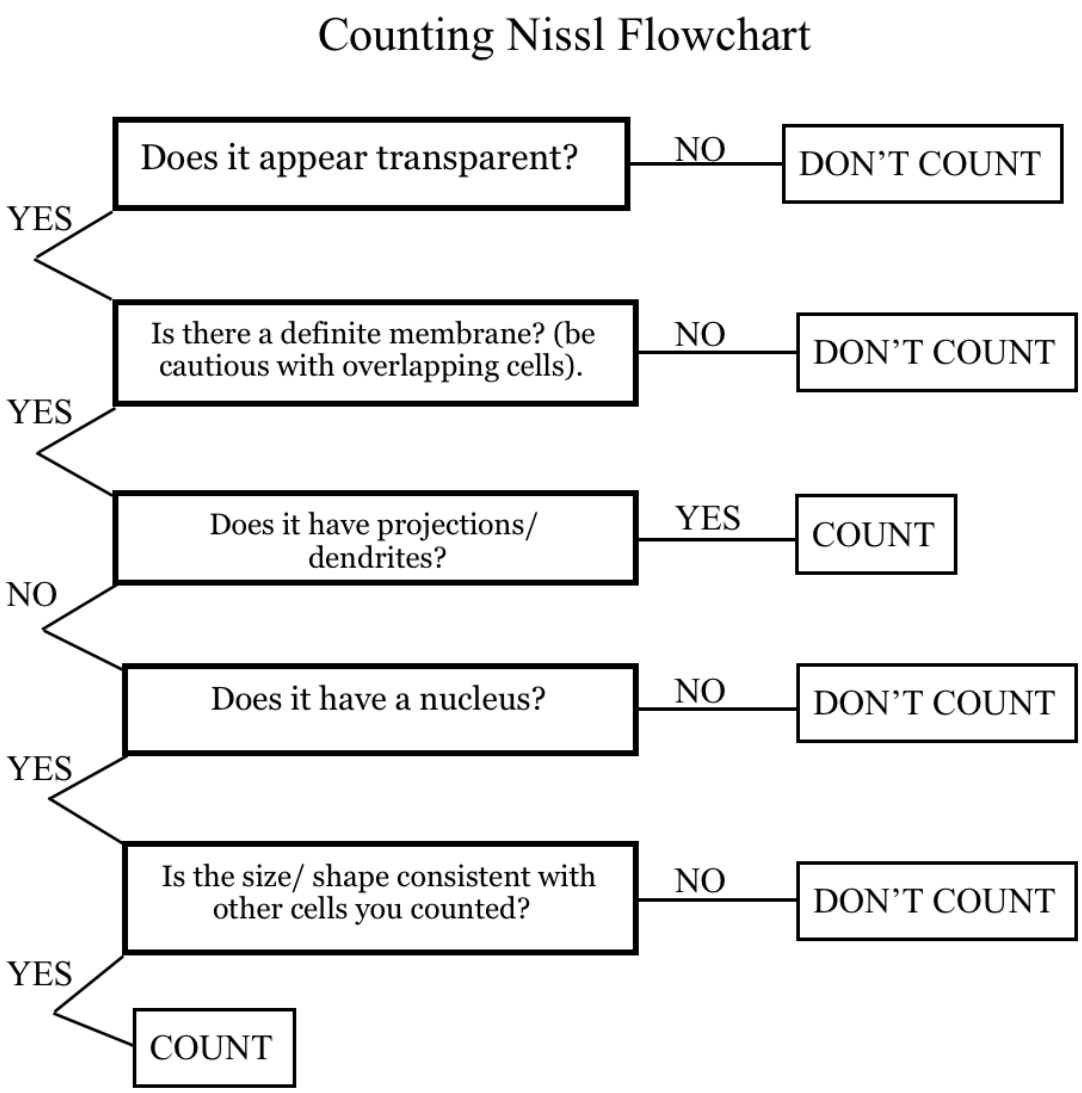

Figure 5. A schematic diagram that shows how thionin stained tissue was counted. The flowchart describes how neurons were identified when counting in Adobe Photoshop. The first question asked about the transparency of the cell. Completely opaque cells represented other glial cells that were not counted. The next question assessed if there was a membrane, an important qualification for identifying a neuron. If the cell had noticeable dendrites or projections, it was counted. If it did not, it would have to have a noticeable nucleus and the cell itself needed to be size and shape consistent with other cells counted in the frame.

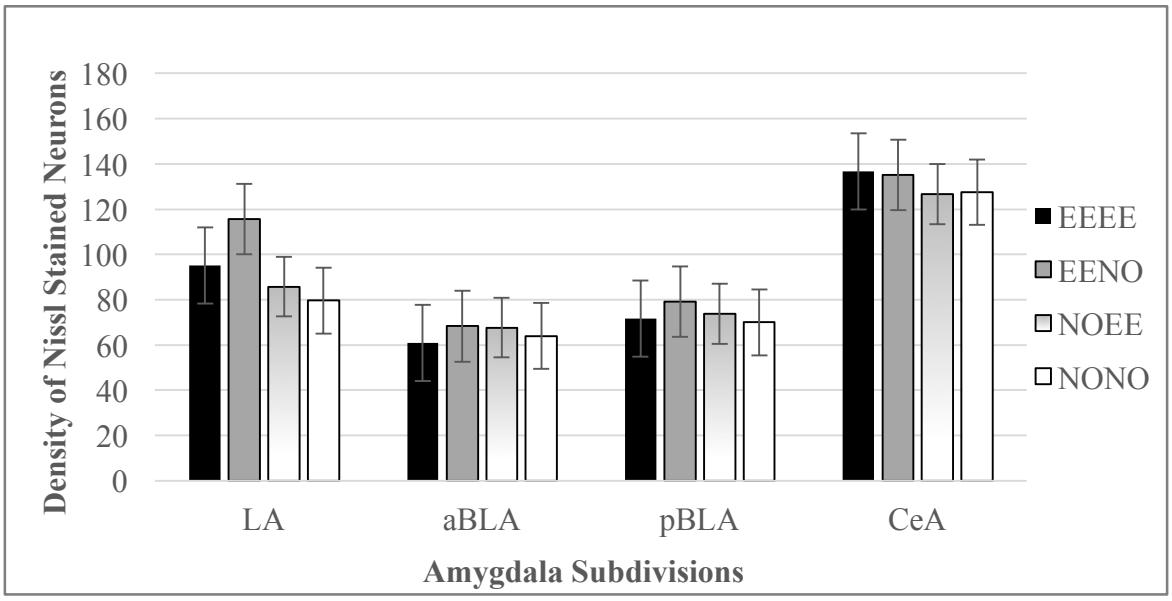

Figure 6. Neuron density with Nissl (per 200 X 200 X 50 μm sample) in Lateral Amygdala (LA), anterior basolateral amygdala (aBLA), posterior basolateral amygdala (pBLA), and central nucleus of the amygdala (CeA) with respect to each experimental group. There was a 24% significant increase of total neuron density in the LA after exposure to a history of EE. The CeA showed the greatest overall density of neurons while the aBLA showed the least density of neurons. Hypothesis tests and specific means are presented in the results section. The error bars represent standard error of mean for each experimental condition and amygdala subdivision. *Abbreviations:* lateral amygdala (LA), anterior basolateral amygdala (aBLA), posterior basolateral amygdala (pBLA), central nucleus of the amygdala (CeA), history of enrichment and last experience of enrichment (EEEE), history of enrichment and no last experience of enrichment (EENO), no history of enrichment but a single last experience of enrichment (NOEE), no history or last experience of enrichment (NONO).

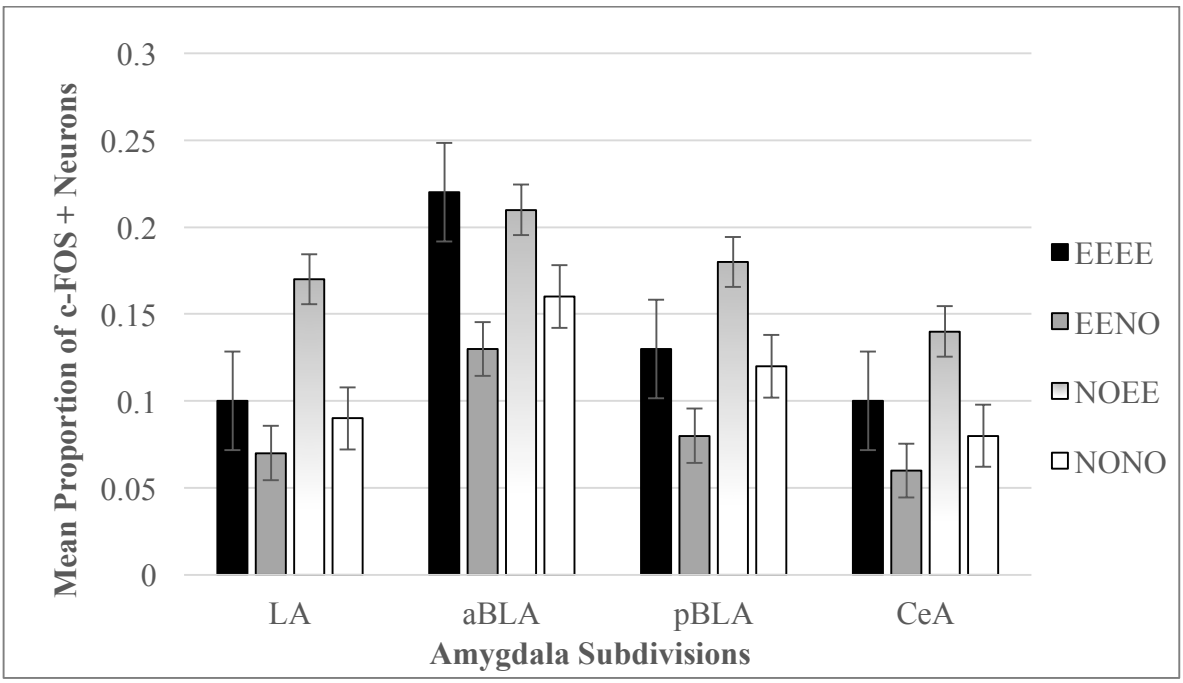

Figure 7. Proportion of c-FOS+ neural activation (per 200 X 200 X 50 μm sample) in Lateral Amygdala (LA), anterior basolateral amygdala (aBLA), posterior basolateral amygdala (pBLA), and central nucleus of the amygdala (CeA) with respect to each experimental group. The proportion of c-FOS+ neurons were calculated utilizing the mean c-FOS+ counts divided by the total neuron density from thionin stained Nissl tissue. This data was analyzed using a 2 X 2 ANOVA to partition variance as well as running t-tests. The error bars represent standard error of mean for each experimental condition and amygdala subdivision. *Abbreviations:* lateral amygdala (LA), anterior basolateral amygdala (aBLA), posterior basolateral amygdala (pBLA), central nucleus of the amygdala (CeA), history of enrichment and last experience of enrichment (EEEE), history of enrichment and no last experience of enrichment (EENO), no history of enrichment but a single last experience of enrichment (NOEE), no history or last experience of enrichment (NONO).

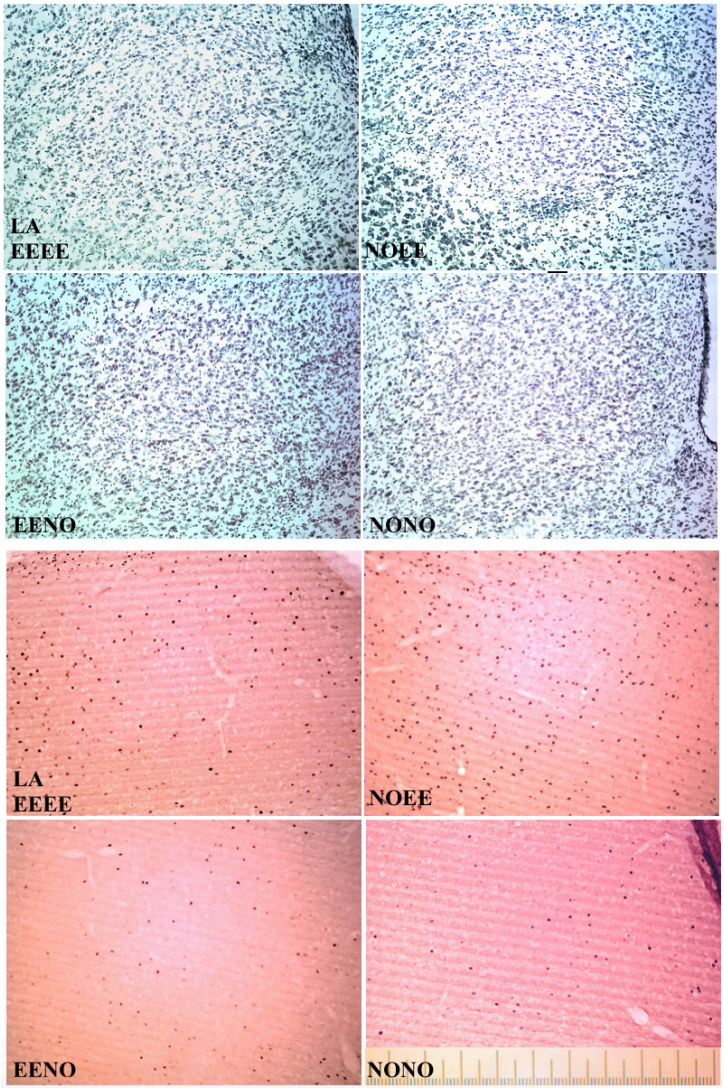

Figure 8. Digital microscopy images of lateral amygdala (LA). Nissl stained tissue is above and c-FOS+ tissue is underneath with experimental groups labeled on the images. There was a 24% increase in neuron density in animals with a history of EE when compared to animals without a history of EE. Proportion of c-FOS+ neurons decreased with a history of EE by 52% compared to no history of EE. The scale bar represents the microns of the image. Each large tic is 100 µm. *Abbreviations:* history of enrichment and last experience of enrichment (EEEE), history of enrichment and no last experience of enrichment (EENO), no history of enrichment but a single last experience of enrichment (NOEE), no history or last experience of enrichment (NONO).

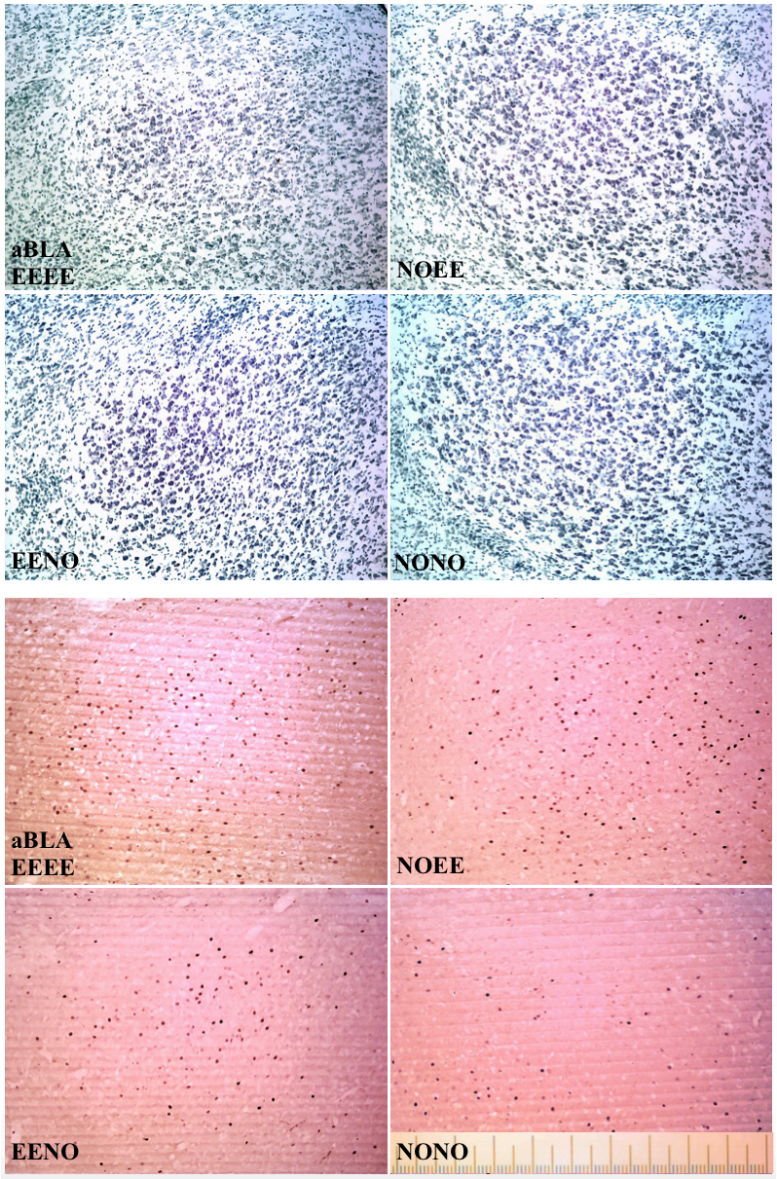

Figure 9. Digital microscopy images of anterior basolateral amygdala (aBLA). Nissl stained tissue is above and c-FOS+ tissue is underneath with experimental groups labeled on the images. The scale bar represents the microns of the image. Each large tic is 100 µm. *Abbreviations:* history of enrichment and last experience of enrichment (EEEE), history of enrichment and no last experience of enrichment (EENO), no history of enrichment but a single last experience of enrichment (NOEE), no history or last experience of enrichment (NONO).

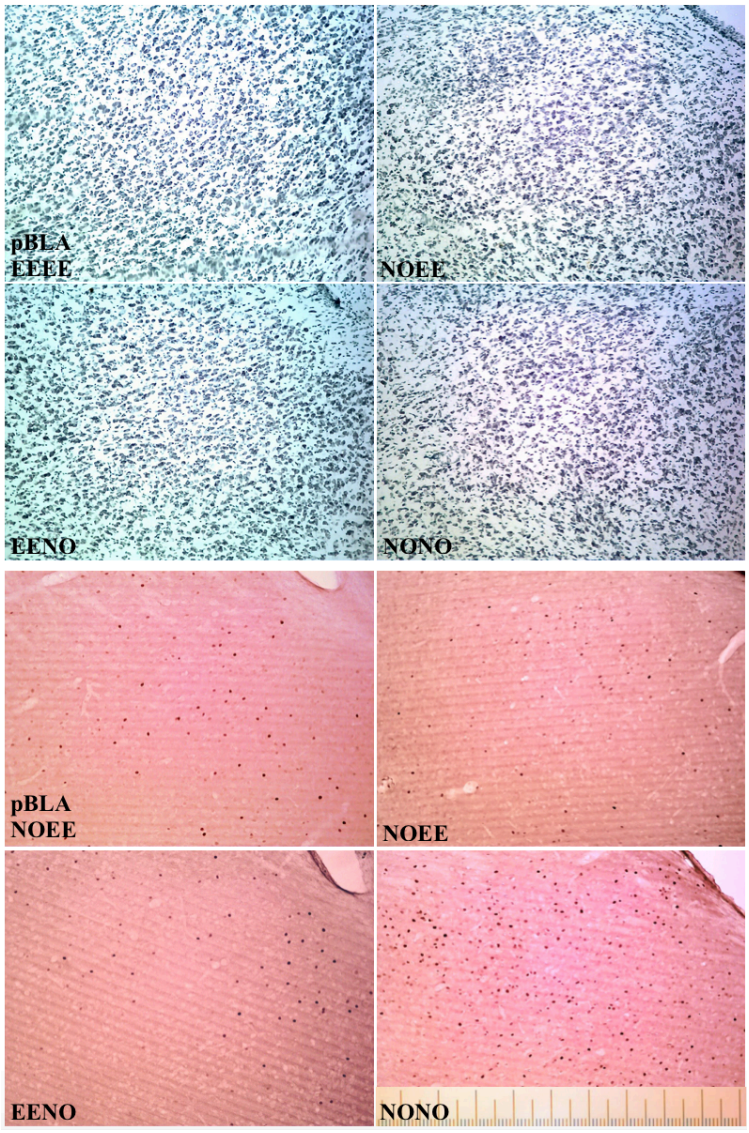

Figure 10. Digital microscopy images of posterior basolateral amygdala (pBLA). Nissl stained tissue is above and c-FOS+ tissue is underneath with experimental groups labeled on the images. Proportion of c-FOS+ neurons decreased with a history of EE by 32% compared to no history of EE. The scale bar represents the microns of the image. Each large tic is 100 µm. *Abbreviations:* history of enrichment and last experience of enrichment (EEEE), history of enrichment and no last experience of enrichment (EENO), no history of enrichment but a single last experience of enrichment (NOEE), no history or last experience of enrichment (NONO).

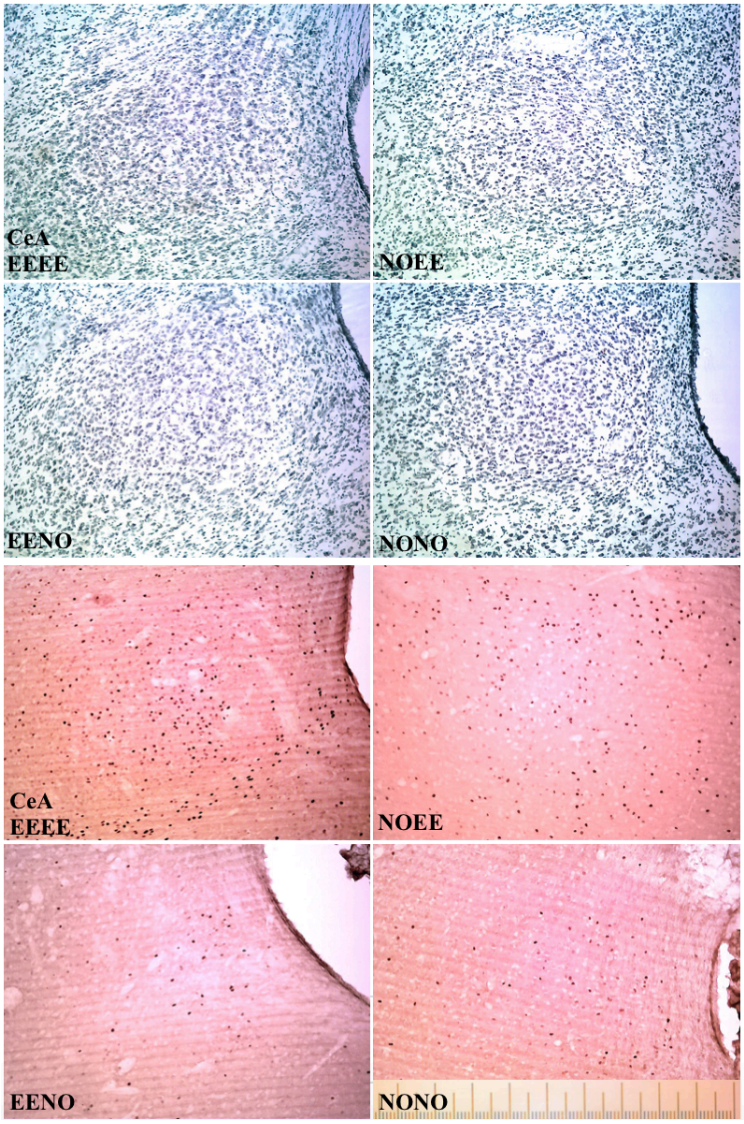

Figure 11. Digital microscopy images of central nucleus of the amygdala (CeA). Nissl stained tissue is above and c-FOS+ tissue is underneath with experimental groups labeled on the images. Proportion of c-FOS+ neurons decreased with a history of EE by 33% compared to no history of EE. The scale bar represents the microns of the image. Each large tic is 100 µm. *Abbreviations:* history of enrichment and last experience of enrichment (EEEE), history of enrichment and no last experience of enrichment (EENO), no history of enrichment but a single last experience of enrichment (NOEE), no history or last experience of enrichment (NONO).

Appendix

TO:	Dr. Mark Zrull Department of Psychology
FROM:	Dr. Ted Zerucha, Chair Institutional Animal Care and Use Committee
DATE:	August 14, 2014
SUBJECT:	Institutional Animal Care and Use Committee Request for Animal Subjects Research
REFERENCE:	*Environmental enrichment, object placement preference, social preference, and associated evoked neural activity in adolescent rats*

IACUC Reference #15-02

Initial Approval Date – August 14, 2014
End of Approval Period – August 13, 2017

The above referenced protocol has been approved by the IACUC for a period of three years.
A list the individuals cleared for research activities with live, vertebrate animals will be sent in a separate email.

Best wishes with your research.

TZ/rst